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Aminergic and Acetylcholinesterase-positive Innervation in the Cerebral Arterial System and Choroid Plexus of the Newt *Triturus pyrrhogaster*, with Special Reference to the Plexus Innervation

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ABSTRACT—The pattern of cerebrovascular noradrenergic or adrenergic (NA) and acetylcholinesterase-positive (AChE) innervation in the newt was investigated. The cerebral arterial tree of this urodelan species was dually innervated by both NA and AChE nerves, with a lesser density of the latter type. NA nerves innervating the major cerebral arteries sometimes originated from NA-containing nerve cells intrinsic to these vessel walls. Another finding worthy of attention was that a large number of NA and AChE nerves were concentrated in the microvascular-epithelial regions of the choroid plexuses, especially the venule loop in the most outer part of the lateral and third plexuses, despite a poor supply of these two nerve types along the entire length of the cerebral arterial tree. This and other findings suggest that NA and cholinergic mechanisms are responsible for the microcirculation and transport action within the choroid plexus in the nutrition of the newt brain via the cerebrospinal fluid. In addition, basophil leucocytes emitted a brilliant greenish yellow fluorescence after formaldehyde gas-treatment. From the combination of the standard excitation and emission spectra and the shifting pattern of these two spectra after HCl vapor treatment, it is expected that specific granules of the newt basophil leucocytes contain not only serotonin and/or catecholamines, but also a substantial amount of fluorescent components other than these biogenic amines and histamine.

INTRODUCTION

It is well established that the major cerebral arteries in mammals are richly innervated by sympathetic noradrenergic and parasympathetic acetylcholinesterase-positive (AChE) nerves with approximately the same density [37]. Dual innervation by these two populations of nerves has also been demonstrated in the choroid plexus, the special vascular-epithelial structure for the secretion of the cerebrospinal fluid (CSF) in the brain ventricles [6, 8, 9, 20, 21].

In urodelan amphibians, Tsuneki and Ouji [34] and Tsuneki et al. [35] demonstrated the absence of blood vessels in the bulk of the brain parenchyma in the Japanese salamanders belonging to the family Hynobiidae. They also pointed out the dense aggregation of brain neuronal perikarya in the periventricular region facing the CSF, the periventricular gray matter, in a variety of urodeles. The urodelan choroid plexus is known to be well vascularized and large relative to the whole brain [13, 17]. Furthermore, a large volume of blood in the brain of this amphibian group has been reported to flow toward the choroid plexus [12]. For these reasons, it has been suggested that the choroid plexus in urodelan amphibians may act as a major site for the supply of nutrients and oxygen to the brain, and for the exit of brain metabolic substances such as carbon dioxide. Thus, it is a matter of interest to explore the neurogenic mechanisms by which the urodelan choroid plexus, as well as their cerebral arterial system, is regulated.

Our previous studies showed a unique pattern of noradrenergic or adrenergic (NA) innervation, and AChE innervation in both the cerebral arterial tree and choroid plexus of some anuran amphibians [2, 31, 32]. The major cerebral arteries of the Japanese toad, the leopard frog, and the bullfrog receive a relatively rich supply of NA nerves , with a poor supply (the former two species) and lack (the latter one) of AChE nerves, while those of the clawed toad have only a few or no aminergic and AChE nerves. Within the choroid plexuses of the Japanese toad, bullfrog, and leopard frog, NA nerves are densely distributed along the arteri al system, but were very poor in the plexus proper consisting of the venule, capillary net and epithelium. In contrast, extremely rich NA innervation has been found in close association with the microvascular-epithelial regions of the choroid plexus in the clawed toad. There are no AChE nerves in the choroid plexuses of these four anuran amphibians, except for a few fibers with very weak enzyme activity in the clawed toad. The present formaldehyde histofluorescence and AChE staining studies on an urodelan species, the newt, are directed

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ACA anterior cerebral artery, AR anterior ramus, BA basilar artery, CCA cerebral carotid artery, FSA first spinal artery, H hypophysis, MCA middle cerebral artery, OC optic chiasma, PCA posterior cerebral artery, PCMT posterior communicating artery, PR posterior cerebral artery, P-L lateral choroid plexus, P-III third choroid plexus, P-IV fourth choroid plexus, TB terminal branch

toward more precise understanding of aminergic and AChE neuronal influence on the choroid plexus as well as the cerebral arterial tree of amphibians.

MATERIALS AND METHODS

Thirty five adult Japanese newts, *Triturus pyrrhogaster*, were used. To clarify the vascular supply of the brain and choroid plexus, five newts were infused through the heart with India ink containing 10% gelatin and placed in ice-cold water. The brain was rapidly removed from the skull and fixed with 10% formalin. Choroid plexuses were then harvested from the lateral, third and fourth ventricles.

Formaldehyde fluorescence and AChE histochemistry

The animals were perfused through the heart with cold Ringer's solution under ethyl ether anesthesia. The brain was removed either immediately, or after perfusion with 4% buffered formaldehyde. The cerebral arterial tree and the choroid plexus were then carefully dissected out from the unfixed or fixed brains. For demonstration of aminergic neurons, the materials obtained from unfixed brains were stretched over nonfluorescent glass slides and transferred to a desiccator to be dried in vacuo over P_2O_5 for 1 h. Air-dried materials were treated with formaldehyde vapor obtained from paraformaldehyde (relative humidity=47%) for 1 h at 80°C [10]. To detect AChE-positive neurons, the arteries and choroid plexuses fixed in formaldehyde for 1 h at 4°C were maintained in Karnovsky's medium without acetylcholine iodide for 30 min at 4°C and then incubated in complete medium containing 2×10^{-4} M tetraisopropyl pyrophosphoramide as an inhibitor of nonspecific cholinesterase activity for 1–5 h at 20°C [18]. The detailed procedures of formaldehyde histofluorescence and AChE staining have been described elsewhere [1].

Microspectrofluorimetry

Microspectrofluorimetric identification of the fluorescent materials induced by formaldehyde gas was carried out using a Nikon SPM-RFL system in accordance with the method of Kojima et al. [19] To differentiate between noradrenaline or adrenaline and two other biogenic amines, dopamine and serotonin, slides were subjected to HCl vapor treatment [3]; the whole-mount preparations on quartz slides were exposed to the vapor of a fresh sample of concentrated HCl solution at room temperature for 1 to 10 min in a closed Petri dish, and then mounted in glycerol under quartz coverslips. All spectra were corrected and expressed as relative quanta versus wavelength.

RESULTS

Angioarchitecture of the cerebral arterial system and choroid plexus

The posterior cerebral artery (PCA) arose from the anterior ramus (AR) to form the anterior circulation of the cerebral arterial tree, together with the middle and anterior cerebral arteries (Fig. 1). On the other hand, the posterior ramus (PR) joined the terminal branches (TB) of the basilar artery (BA) at the midline of the upper part of the medulla oblongata, and formed the posterior circulation. At the level where the right and left PR ran caudally along the

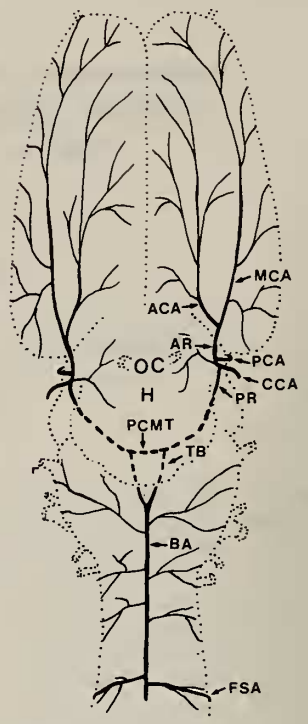

Fig. 1. Diagram of arterial supply to the brain of the newt (ventral view).

caudal portion of the hypophysis, they communicated through the posterior communicating trunk (PCMT). No anterior communicating artery was found between the right and left anterior circulations, so that the circle of Willis was not complete.

The choroid plexuses from the lateral and third ventricles (P-L, P-III) were large in proportion to the brain size, and appeared with a butterfly wing-like profile (Fig. 2). The lateral and third choroidal arteries arising from the PCA ramified over the plexus proper, so that they made up a fine and well-developed capillary network between the ependymal (epithelial) cell layer and stroma. The outermost part of the P-L and P-III were fringed with the venule lining. The choroid plexus from the fourth ventricle (P-IV) was triangular in shape, and was supplied by branches from both the PR and the BA.

Formaldehyde fluorescence

Aminergic innervation

A few thick fluorescent fiber bundles, which entered the cranial cavity along the internal carotid artery (ICA) via the carotid canal, ran longitudinally along the AR or PR toward the anterior or posterior circulations (Fig. 3AB). Such fluorescent fiber bundles were also seen to ascend along the wall of the caudal part of the BA via the first spinal artery (FSA) which corresponds to the vertebral artery in mammals, and to meet with the descending fiber bundles via the ICA around the TA (Fig. 3CD). A small number of thin fibers emanated from fluorescent fiber bundles and were distributed spirally or circularly along the major cerebral arteries of the

FIG. 2. Photomicrograph of the lateral (L) and third (III) choroid plexuses. C: Capillary net. Arrows indicate the venule lining running along the plexus outer margin. ×18.

anterior and posterior circulations with approximately equal density (Table 1). Interestingly, a few nerve cells emitting a strong greenish yellow-fluorescence, which showed a simple multipolar or pseudomultipolar profile, were situated singularly on the walls of the cerebral carotid artery (CCA), the intracranial part of the ICA, and the AR in some individuals (Figs. 3B). Their axons ran parallel to, or spirally toward the major arteries of the anterior circulation. Some of these also descended towards the posterior circulation, and could be followed up to the rostral part of the BA.

The innervation density of aminergic nerves in the choroid plexuses from all the ventricles was significantly higher compared to that observed in the major cerebral arteries (Fig. 4A, Table 1). A few fluorescent fiber bundles on the lateral and third choroidal arteries, which branched out from thick fiber bundles on the PCA via the CCA, spread radially when they entered the corresponding choroid plexuses, and built up a well-defined and dense network of thin varicose fibers over the whole of the microvascular-epithelial region. The nerve supply was much more prominent along the venule loop in the outermost part, in comparison to the capillary net (Fig. 4B). The density of aminergic nerves in the P-IV was approximately the same as that observed in the P-L and P-III. There were no fluorescent nerve cells in any choroid plexuses examined.

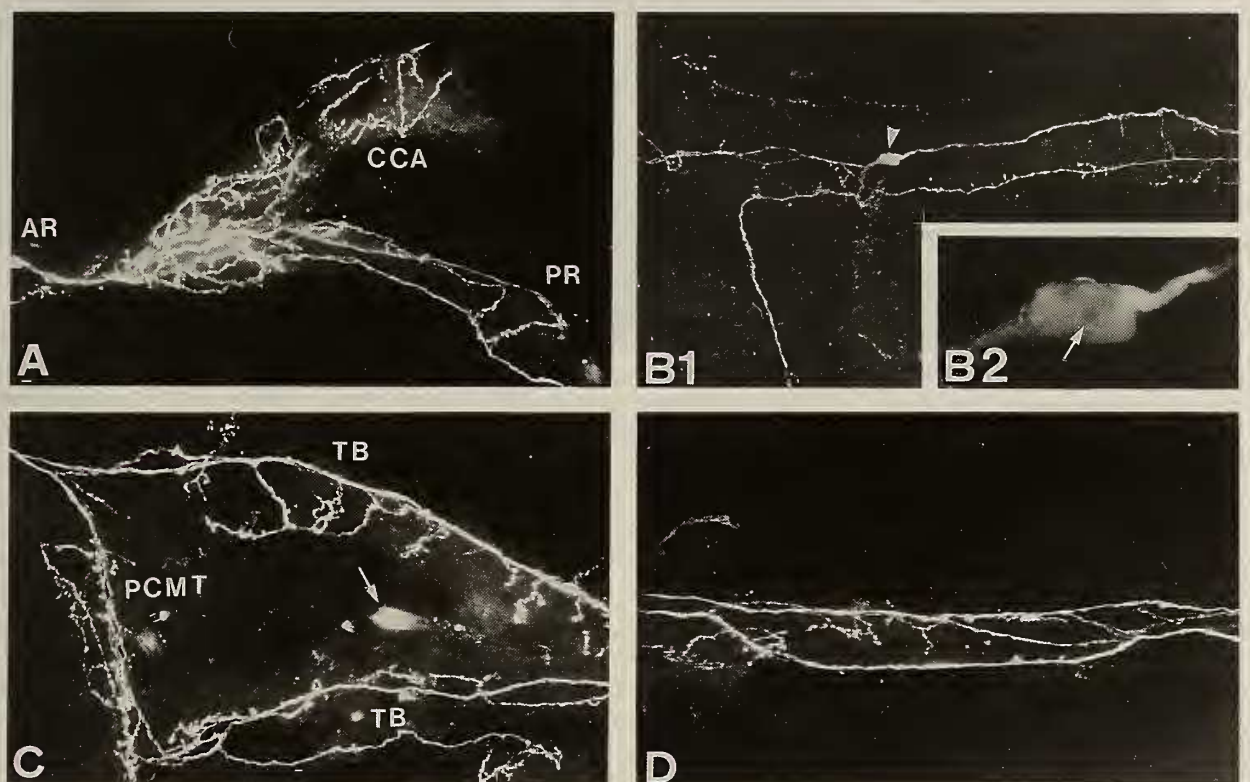

FIG. 3. Fluorescence photomicrographs of whole-mounts showing (nor) adrenergic innervation in the major cerebral arteries. A: Cerebral carotid artery (CCA), and anterior and posterior rami (AR, PR). B: Anterior ramus. Arrowhead indicates single aminergic nerve cell located at the wall of the AR, and arrow indicates its nucleus. C: Posterior communicating trunk (PCMT) and terminal branch (TB). Arrow indicates basophil leucocyte. D: Basilar artery. A, C, D ×165; B1 ×100; B2 ×500.

TABLE 1. Density of NA and AChE nerves in the major cerebral arteries and choroid plexuses of the newt

	Arteries								Choroid plexuses		
	ACA	MCA	PCA	AR	CCA	PR	TB	BA	P-L	P-III	P-IV
NA	+	+	+	+	+	+	+	+	++	++	++
AChE	+	+	+	+	+	+	+−	+−	++	++	+

The relative number of nerve fibers was graded arbitrarily. − absent; + a few fibers; ++ dense fibers.

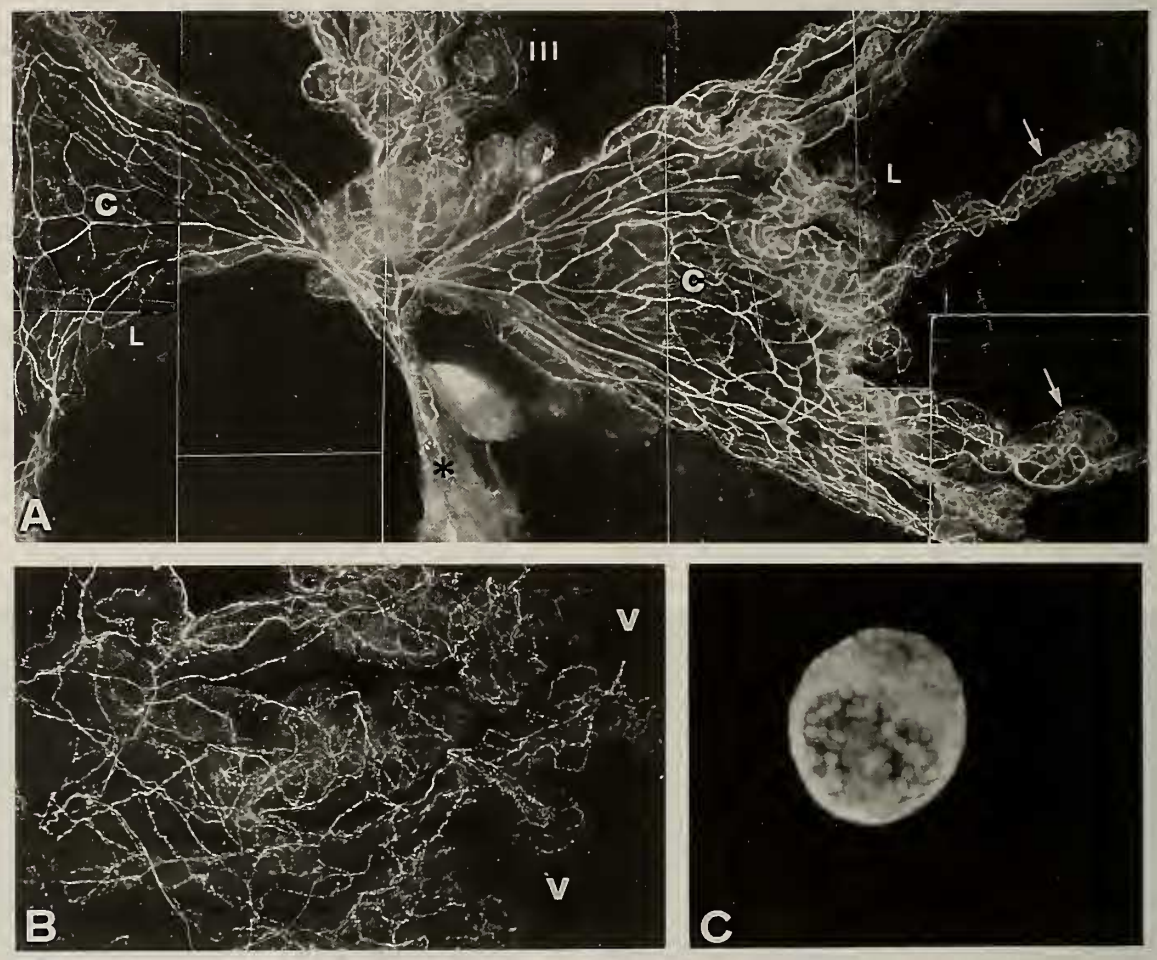

FIG. 4. Fluorescence photomicrographs of whole-mounts showing (nor)adrenergic innervation in the choroid plexuses (A, B), and of a blood smear showing fluorescent basophil leucocytes (C). A: Lateral (L) and third (III) choroid plexuses, capillary net (C). Arrows indicate venule lining, and arrowhead indicates basophil leucocyte. Asterisk indicates choroidal artery arising from the posterior cerebral artery. B: Venule lining of the third choroid plexus and ventricle (v). C: Basophil leucocyte. A ×66; B ×132; C ×500.

Basophil leucocyte

Specific granules filling the cytoplasm of the basophil leucocytes emitted a brilliant greenish yellow-fluorescence after treatment with formaldehyde gas for 1 h (Fig. 4C), but did not show such fluorescence without formaldehyde treatment. A longer irradiation with ultraviolet light, as was also the case with water treatment, resulted in a striking extinction of fluorescence.

Microspectrofluorimetric analysis

The excitation and emission spectra of the greenish-yellow fluorescent ganglion cells and nerves showed large peaks at about 410 and 480 nm, respectively, with a small peak in the excitation spectrum at 330 nm (Fig. 5A). After exposure to HCl vapor for 5 min or more, the maximum peak of the excitation spectrum shifted to 330–340 nm, concomitant with a striking lowering of the peak at 410 nm, while the emission maximum was remained unchanged. During the course of HCl treatment, the fluorescence faded very rapidly. This is a typical pattern characteristic of adrenaline or noradrenaline [3].

The standard excitation spectrum of fluorescent basophil leucocytes showed a large peak at about 415 nm and a small peak at about 330 nm, and the emission spectrum had a broad

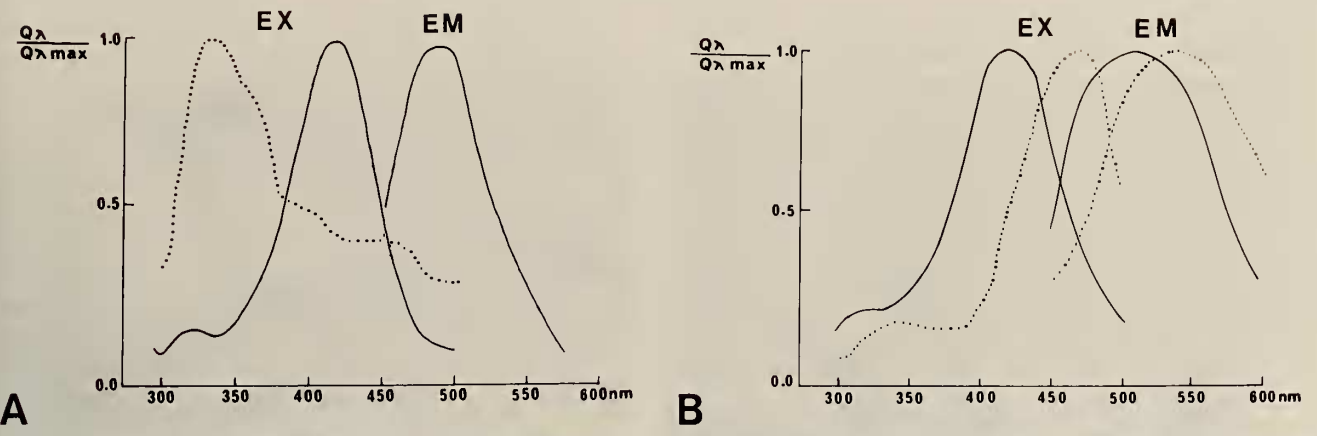

FIG. 5. Excitation (EX) and emission (EM) spectra of fluorescent nerve cells in the walls of the anterior ramus (A) and of fluorescent basophil leucocytes (B). —: after formaldehyde gas treatment only; ---: after formaldehyde gas treatment followed by treatment with HCl vapor for 5 min or more. All spectra were corrected and expressed as relative quanta.

peak ranging from 495 to 515 nm (Fig. 5B). After HCl vapor treatment, the greenish-yellow fluorescence changed into a yellowish color with no detectable level of fading. Both the large and small peaks on the excitation spectrum shifted to about 480 and 350 nm, respectively, and the maximum emission peak also shifted to around 520–540 nm.

AChE staining

A few thick fiber bundles that were stained intensely for AChE reaction were consistently present on the CCA (Fig. 6A), but none were found in the walls of the FSA to BA. AChE fiber bundles on the CCA ran rostrally and caudally towards the anterior and posterior circulations. The density of AChE nerves in the major cerebral arteries appeared to be lower, particularly along the BA, than that of NA nerves (Fig. 6, Table 1), although it was difficult to follow the precise distribution pattern owing to the high level of non-nervous AChE-activity in the vessel walls themselves except for the wall of the CCA.

AChE-positive nerves supplying the P-L and P-III also showed high-level activity of this enzyme, and were apparently rich compared to those supplying the P-IV and the major cerebral arteries. These positive nerves were distributed in a manner similar to NA nerves (Fig. 6D, Table 1): the axons from AChE fiber bundles on the choroidal stem arteries formed a well-developed meshwork of thin fibers over the microvascular-epithelial regions, with a preference for the venule lining in the outermost part, rather than for the capillary-epithelial complex. There were no ganglionic structures positive for AChE in the choroid plexuses from all the ventricles, as well as in the walls of the major cerebral arteries in all parts of the brain.

DISCUSSION

Species differences

The present study documented for the first time the innervation pattern of NA and AChE nerves in the cerebral arterial tree and choroid plexus of the newt. The supply of these two types of cerebral perivascular nerves to the major cerebral arteries of this urodele, which is characterized by a lesser density of AChE nerves, is less rich than those of the Japanese toad and leopard frog [31, 32], but is not as poor as that of the clawed toad [2]. In contrast, the innervation of choroid plexus by NA nerves in the newt is distinctly denser than that observed in the Japanese toad, bullfrog, and leopard frog [31, 32], although it is not as prominent as that of the clawed toad [2]. The P-L and P-III of this urodelan species also have a richer AChE innervation comparable in density to the NA innervation, receiving the most abundant supply of AChE nerves of the various amphibian choroid plexuses investigated to date. The distribution of NA and AChE nerves projecting to the newt choroid plexus, unlike the NA innervation in the Japanese toad and bullfrog, but like that in the clawed toad [2], is predominant in both the microvascular system and epithelium, particularly along the venule loop in the P-L and P-III. Thus, each of the amphibian species investigated has its own characteristic pattern of plexus innervation by NA and AChE nerves that is quite different from the innervation pattern seen in the major cerebral arteries.

Source and pathway of aminergic and AChE innervation

It is well known that sympathetic ganglia typical of higher vertebrates are not present in the cyclostomes, the most primitive vertebrate, but many aminergic (serotonin-containing) nerve cells are scattered over, or clustered along the cardiovascular system [27]. Indeed, the major cerebral arteries of the lamprey, a member Cyclostome species, are innervated by serotonergic nerve cells intrinsic to these vessel walls [15]. In most cases in newts, NA nerves innervating the major cerebral arteries are all of extracranial origin (presumably from cervical sympathetic chain ganglia) as reported in anuran amphibians [2, 31, 32], and pass through the ICA and FSA to reach the cranial cavity. However, we have demonstrated that a few NA nerve cells occur on the

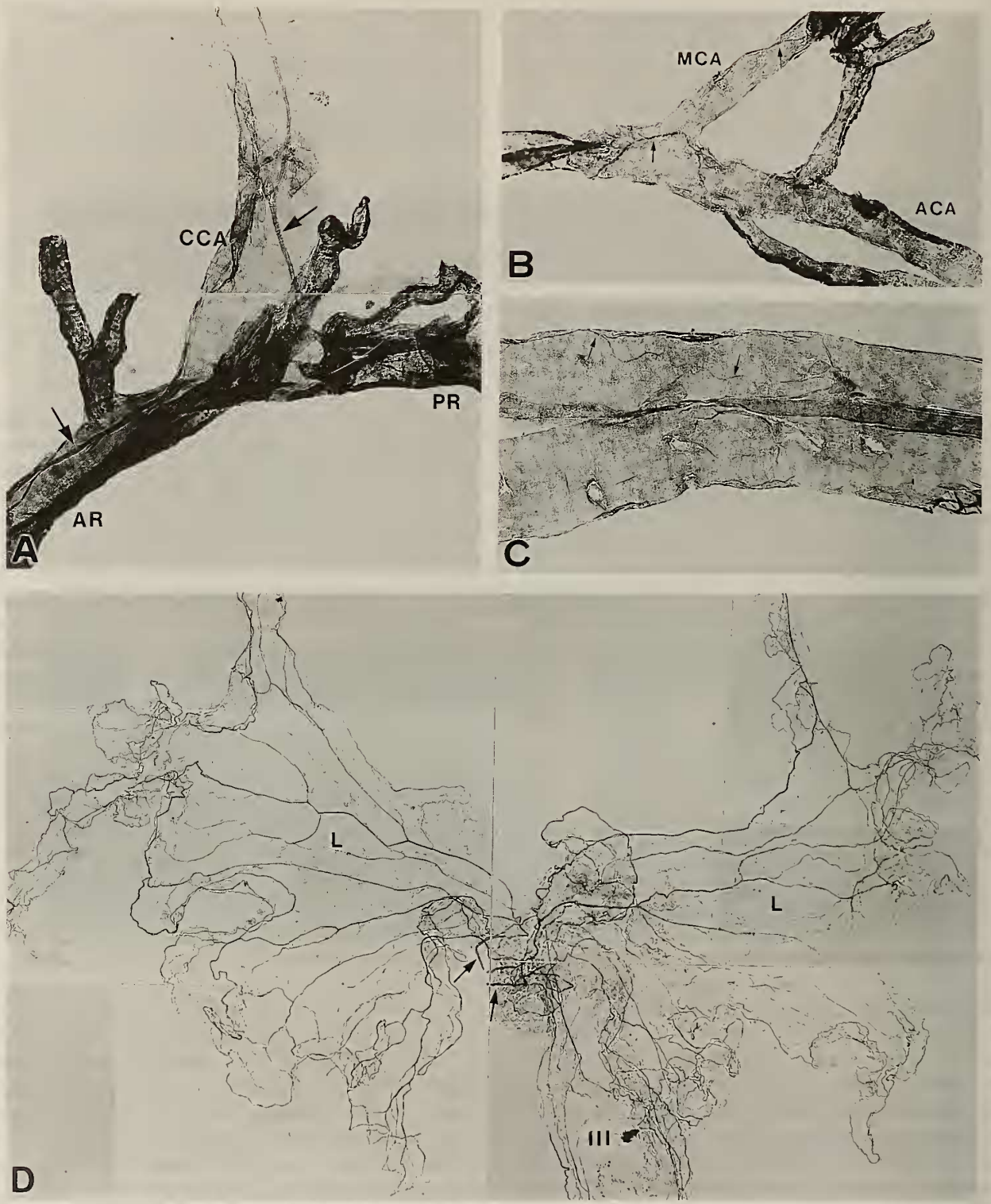

Fig. 6. AChE staining in whole-mounts of the major cerebral arteries (A, B and C) and choroid plexuses (D). A: Cerebral carotid artery (CCA) and anterior and posterior rami (AR, PR). B: Middle and anterior cerebral arteries (MCA, ACA). C: Terminal branch of the basilar artery. Large arrows indicate thick fiber bundles positive for AChE. Small arrows indicate AChE nerve fibers. Note a high non-nervous AChE activity in the walls of the major cerebral arteries. D: Lateral (L) and third (III) choroid plexuses. Arrows indicate AChE fiber bundles on the choroidal stem arteries that extend over the plexus proper. A, B, C ×125; D ×50.

walls of the major cerebral arteries in some individuals, and some of the fluorescent nerves in fact originate there. Such a primitive profile of aminergic innervation that is maintained even now by the newt cerebral circulation emphasizes further the concept of amphibian phylogeny that urodelan amphibians are much more primitive than anurans.

The present study has clearly shown the wide spread of NA axons within fluorescent fiber bundles which run along the choroidal stem arteries via the ICA or FSA over the choroid plexus in the newt. Similar sources and vascular pathways of extracranial NA nerves for plexus innervation have also been noticed in the Japanese toad and bullfrog [2]. However, this pattern cannot be regarded as a common feature of the plexus NA innervation in amphibian, since our previous study revealed that NA nerves within the choroid plexuses of the clawed toad originate in the axons from NA ganglion cells located at the plexus stroma [2]. Furthermore, the possibility that the axons from NA nerve cells intrinsic to the major cerebral arteries may contribute, to some extent, to the plexus innervation cannot be excluded in some newts.

In the newt, AChE fiber bundles, unlike the results of formaldehyde histofluorescence, were found only along the ICA. This finding, in addition to the lack of nerve cells positive for AChE throughout the pial vasculature and choroid plexus, seem to indicate that AChE nerves supplying the cerebral arterial tree of this urodelan species come from the ICA alone, and then extend to the choroid plexus via the corresponding choroidal arteries in a manner preferential for the P-L and P-III. Although it is difficult to make an exact identification between parasympathetic cholinergic neurons and sympathetic or sensory neurons soley by AChE staining, it is generally accepted that high concentrations of AChE are located in parasympathetic cholinergic neurons, and low levels of the enzyme activity are associated with sympathetic or sensory neurons [29]. AChE nerves contributing to the rich innervation of the newt choroid plexus, as well as the fiber bundles present on the ICA, were stained strongly with this enzyme reaction, and thus are probably parasympathetic in nature.

Functional implications

No neurogenic influence on choroid plexus function has been reported for amphibian. However, the two major effects of NA nerves on plexus vascular-epithelial function, inhibition of CSF production by the epithelial cells, and manipulation of active transport between the CSF compartment and blood, have been shown in mammals [5, 7, 11, 22–26]. In addition, AChE nerves have been suggested to exert vasodilatory effects in the cerebral circulation [4]. Therefore, the rich innervation of NA and AChE nerves focused on the microvascular system and epithelium within the choroid plexus in the newt, despite a less rich supply of the two nerve types along its cerebral arterial tree, must be considered in relation to the secretomotor and transport actions essential for the functioning of these plexus elements. Since the critical role of the choroid plexus for the brain nutrition and metabolism via the CSF has been proposed on the basis of the brain structures characteristic of urodelan amphibians including the newt [28, 34, 35], it might deeply participate in the regulation of such plexus functions. In this regard, distinct species differences in the innervation density and regional distribution of NA and AChE nerves in amphibian choroid plexuses may involve species-specific NA and cholinergic mechanisms responsible for CSF production from epithelial cells, and the exchange of oxygen, nutrients and metabolic products between this special vascular-epithelial structure and brain parenchyma.

Basophil leucocytes

The specific granules of basophil leucocytes of the newt, which comprise about 48% of total leucocytes [16] and contain only a trace amount of histamine [33], emitted brilliant greenish-yellow fluorescence after exposure to formaldehyde gas. The standard excitation (EX) and emission (EM) spectra of these fluorescent granules were different from those of the formaldehyde gas-induced fluorescence in the turtle basophil leucocytes (EX/EM max: 470/495 nm) [14] and in the histamine (EX/EM max: 380/450 nm) [36]. The findings presented here suggests that the specific granules in the newt basophil leucocytes mainly consist of serotonin and/or catecholamines (dopamine, noradrenaline, adrenaline), considering the remarkably high fading rate of fluorescence after water treatment and irradiation with ultraviolet light. However, the shifting pattern of EX and EM spectra after HCl vapor treatment, which did not coincide with those of serotonin (EX/EM max: 390/530 nm), dopamine (EX/EM max: 320 and 365/490 nm), or (nor) adrenaline (EX/EM max: 320/490) [3, 30], leads to the speculation that the newt basophil leucocytes also contain substantial amounts of fluorescent components other than these biogenic amines. Further biochemical research must be carried out before any firm conclusion can be made on this matter.

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